N-170: Large Scale Biomass Production of Obligate Anaerobes for Simultaneous Transcriptomics, Proteomics, Metabolomics, and Lipidomics Analysis

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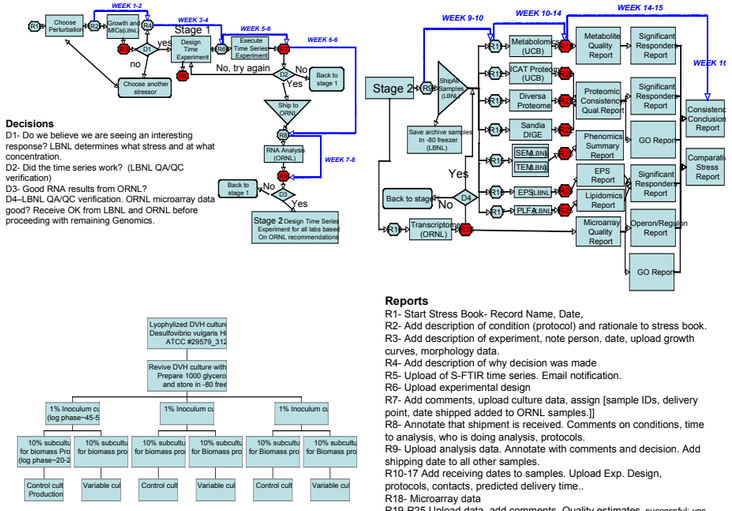
Abstract

To rapidly determine induced stress response pathways in anaerobic microorganisms, we need to produce biomass for simultaneous analyses using the latest techniques in transcriptomics, proteomics, metabolomics, and lipidomics. To accomplish this, batch cultures of 30 liters of stressed vs. non-stressed *Desulfovibrio vulgaris* as biological replicates in triplicates are needed to ensure that all the analyses will be performed on cells of same condition. Various technical improvements and adaptations were made for the large-scale production and distribution of biomass exposed to a variety of stressors, such as oxygen, salt, nitrate, nitrite, and temperature. Harvesting of *D. vulgaris* through high-speed centrifugation proved to be the most efficient and uniform method of collecting cells for the various types of analyses. Because of the rapidly changing nature of DNA and the short half-life of mRNA, *D. vulgaris* cultures needed to be immediately cooled to 5°C during biomass sampling. As a result, a fast sample cooling system was developed to chill 300 ml of biomass culture from 30°C to 5°C in less than 3 min at a flow rate of 1.8 ml/s. For the oxygen stress of *D. vulgaris*, culture vessels were fitted with HPLC three-valve delivery caps and porous teflon tubing filled with beads for sparging of the cultures with different types of gases (O_2 & N_2) at various concentrations in a controlled manner. Because of the concomitant analysis by several laboratories, rigorous quality control measures were used to insure the quality and sterility of biomass from each time point in a production run, e.g. direct cell counts, optical density, pH, plate streaks, phospholipid fatty acid (PLFA) analysis, and protein assays. In addition, advanced FTIR spectromicroscopy profiling was used to study gross bimolecular changes and to determine optimal sampling times. QA/QC procedures were developed and documented to track every step in production from experiment inception to final analyses, including all chemicals, procedures, and technicians. Data are immediately uploaded to a database that is shared by all investigators (http://vimss.lbl.gov)

Introduction

The Virtual Institute for Microbial Stress and Survival (VIMSS) at Lawrence Berkeley National Laboratory (LBNL) seeks to identify stress response pathways of *Desulfovibrio vulgaris* induced by various environmental factors by combining multiple simultaneous analyses in an effort to conceptualize these pathways. The Applied Environmental Microbiology core at LBNL is responsible for producing large quantities of *D. vulgaris* biomass for different research laboratories for simultaneous analyses on cells with the same growth condition and stress level. Transcriptomics (K-123) are studied at Oak Ridge National Laboratory (ORNL); proteomics are studied at UC Berkeley, Sandia National Lab (H-087), and Diversa Corporation. Metabolomics are conducted at UC Berkeley; lipidomics (N-184) and phenomics (Q-247) are studied at LBNL. Biomass production at LBNL involves the production of non-stressed and stressed *D. vulgaris* culture at various time points during the growth phase. It is a four-month process with two major stages. During stage one, LBNL determines the stressor and the dosage, and designs and carries out a multi-time point experiment for ORNL's transcriptome analysis. During stage two, LBNL will produce 12 to 30 liters of biomass for all the VIMSS laboratories based on the results of transcriptomics. LBNL is also responsible for all QA/QC verifications, all sample shipments, the uploading of data, and analysis for all experiments.

Biomass Production and VIMSS Pipeline



Decisions
D1- Do we believe we are seeing an interesting response? LBNL determines what stress and at what concentration.
D2- Did the time series work? (LBNL QA/QC verification)
D3- Good RNA results from ORNL?
D4–LBNL QA/QC verification. ORNL microarray data good? Receive OK from LBNL and ORNL before proceeding with remaining Genomics.

Figure 1. Flow chart describing a standard biomass production experiment with biological replicates: three controls and three variables

Reports
R1- Start Stress Book- Record Name, Date,
R2- Add description of condition (protocol) and rationale to stress book.
R3- Add description of experiment, note person, date, upload growth curves, morphology data.
R4- Add description of why decision was made
R5- Upload of S-FTIR time series. Email notification.
R6- Upload experimental design
R7- Add comments, upload culture data, assign [sample IDs, delivery point, date shipped added to ORNL samples.]]
R8- Annotate that shipment is received. Comments on conditions, time to analysis, who is doing analysis, protocols.
R9- Upload analysis data. Annotate with comments and decision. Add shipping date to all other samples.
R10-17 Add receiving dates to samples. Upload Exp. Design, protocols, contacts, predicted delivery time..
R18- Microarray data
R19-R25 Upload data, add comments, Quality estimates, successful: yes or no, requests for special analysis, etc. Automate analyses delivered back to stress book.

QA/QC Verifications

- Colony and cell morphology - Purity streak and Microscopy
- Fluorescence antibody stain for presence of DVH
- Synchrotron Fourier Transform Infrared Spectromicroscopy (S-FTIR)
- Transmission Electron Microscopy (TEM)
- Scanning Electron Microscopy (SEM)

Expt#; Timepoint#; Replicate# (e.g. E26T0C1)	OD (600nm)	AODC (cells/ml)	PLFA (cells/g)	Proteins (ug/ml)	pH	Aerobic streak (TSA plate)	Anaerboic strea (LS4D plate)

Results

Over a twenty-month period from September 2003 to May 2005 the Applied Environmental Microbiology core at LBNL conducted over forty large scale *D. vulgaris* biomass production experiments and countless small scale experiments. During this time, the group developed various techniques and made numerous improvements to VIMSS biomass production such as in media composition, anaerobic sampling, and biomass harvesting. Direct filtration and tangential flow filtration were studied as viable cell harvesting alternatives to centrifugation. However, studies showed that centrifuging *D. vulgaris* cells was the best option in terms of time, cost, efficiency, and quality control (Figure 3). The accumulation of optical density (OD), Acridine Orange Direct Cell count (AODC), protein, and PLFA data over time have enabled an accurate prediction of *D. vulgaris* growth rate. The consistency of growth determined by the comparison data allows a set biomass production schedule and sampling time, which minimize variability between experiments.

Figure 2. Lactate-sulfate defined (LS4D) medium recipes

Trace Minerals Stock solution	g/L	Molarity	Thiamyn Vitamins Stock solution	g/L	Molarity
Nitrilotriacetic acid	12.8	30 mM	d-Biotin	0.02	82 µ M
$FeCl_2 \cdot 4 H_2O$	1	5.0 mM	Folic Acid	0.02	45 µ M
$MnCl_2 \cdot 4 H_2O$	0.5	2.5 mM	Pyridoxine Hydrochloride	0.1	490 µ M
$CoCl_2 \cdot 6 H_2O$	0.3	1.3 mM	Thiamine Hydrochloride	0.05	150 µ M
$ZnCl_2$	0.2	1.5 mM	Riboflavin	0.05	130 µ M
$Na_2MoO_4 \cdot 2 H_2O$	0.03	220 µ M	Nicotinic Acid	0.05	410 µ M
H_3BO_3	0.02	320 µ M	Pantothenic Acid	0.05	210 µ M
$NiSO_4 \cdot 6 H_2O$	0.1	380 µ M	p-Aminobenzoic Acid	0.05	310 µ M
$CuCl_2 \cdot 2H_2O$	0.00 2	70 µ M	Thioctic Acid	0.05	240 µ M
Na_2SeO_3	0.00 2	30 µ M	Choline Chloride	2	14 mM
$Na_2WO_4 \cdot 2H_2O$	0.00 4	20 µ M	Vitamin B12	0.05	7.4 µ M

Compound	mM media	Molarity in Medium
MOPS-Q water	100 mL	100 mL
1M Sodium sulfate	30 ml	30 mM
2M Sodium lactate	30 ml	60 mM
1M Magnesium chloride	8 ml	8.0 mM
4M Ammonium chloride	5 ml	20 mM
1M Potassium phosphate	2.2 ml	2.2 mM
1M Calcium chloride	0.6 ml	0.6 mM
Trace minerals	12.5 ml	
1M PIPES buffer	30 ml	30 mM
Resazurin (0.1%)	0.16 ml	0.016 µ M
5% Sodium Hydroxide	1 ml	10 mN
Thiamin vitamins	1 ml	
Titanium Citrate	5 ml	

	Centrifugation	Direct filtration	Tangential Filtration
Approxmiate Processing time (for 6 samples)	~10 min.	~30-45 min.	~50-80 min.
Estimated Processing costs	~$150	~$60	~$400-$800

Figure 3. Biomass harvesting methods comparison chart

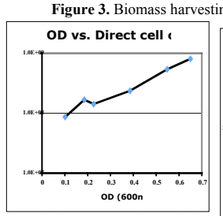


Figure 4. OD vs. AODC. Prediction of approximate cells/ml based on OD readings

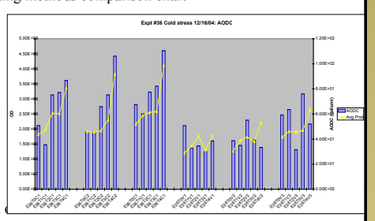


Figure 5. Proteins vs. AODC data from Experiment #36: cold stress

Fast Sample Cooling System for Biomass Production

During biomass production, six *D. vulgaris* cultures each in two to five liter bottles are grown at 30°C in the anaerobic chamber and sampled at various time points during the growth phase. However, due to the short half life of mRNA, proteins , and metabolites, it is necessary to rapidly chill large quantities of biomass samples to 4-5°C within seconds in order to stop cell growth and be able to determine the effect of different stressors on *D. vulgaris* over time. Samples on ice and centrifuging the samples at 4°C will not adequately chill the samples fast enough to prevent cells from growing and changes to DNA and mRNA during sample processing. A fast sample cooling system was developed to sample *D. vulgaris* cultures in the anaerobic chamber and chill large volumes of culture (300-600 ml) from 30° to 4°C immediately (Figure 6). The sample apparatus uses a peristaltic pump and sterile Masterflex viton tubing to remove *D. vulgaris* samples from the culture bottles. The culture chills almost immediately as it passes through chilled sterile coiled stainless steel tubing and into the receiving bottle (Figure 7). The stainless steel tubing and receiving bottles are both placed in ice and remain chilled during the process. Cooling tests confirmed that *D. vulgaris* samples can be sampled quickly and chilled in a sufficiently short time. Three hundred milliliters of *D. vulgaris* culture can be sampled in less than three minutes and is chilled almost immediately. Results from the cooling tests can be seen in Figure 8.

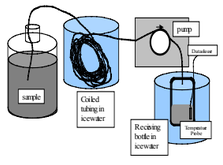


Figure 6. Rapid sample cooling system for biomass production

Figure 7. *D. vulgaris* culture pumped through ice chilled stainless steel tubing with a peristaltic pump

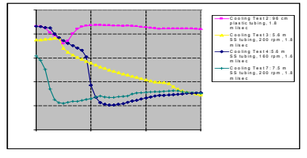


Figure 8. Cooling test results. Stainless steel tubing chilled *D. vulgaris* culture better than plastic tubing. The use of 7.5 m stainless steel tubing and a pump rate of 200 rpm resulted in the rapid cooling of *D. vulgaris* samples in a sufficiently short sampling time (1.8 ml/sec or 108ml/min).

Sparge cap system for Oxygen Stress Experiments

HPLC three valve solvent delivery caps and white porous teflon tubing filled with beads were used for oxygen stress experiments to maximize gas saturation efficiency. Using the sparge cap system, oxygen saturation in culture and in media were achieved in less than 30 minutes. Different concentrations of oxygen can be sparged and introduced to *D. vulgaris* culture in a controlled manner.

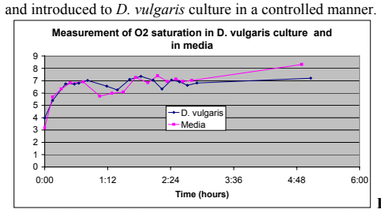


Figure 9. Graph of dissolved oxygen measurements in culture and in media over time

Figure 10. Sparge cap system used for oxygen stress experiments

Freezer chill packs

Freezer chill packs and ice were used to keep the samples cool after sampling. Freezer chill packs cut to properly surround and chill sampling bottles and falcon tubes.

Figure 11. Freezer chill packs cover sampling bottles and tubes

VIMSS Online Lab Notebook/Database

For every experiment, there is a virtual lab notebook documenting all QA/QC data. Every step in biomass production from the beginning of media preparation to the shipment of samples in the end is documented in an excel spreadsheet and uploaded online to be made available to all VIMSS researchers.

Figure 12. VIMSS online database at http://vimss.lbl.gov and a list of biomass production experiments available online.

Conclusions and Future Work

- Biomass production of batch cultures in biological replicates demonstrated a reliable and carefully controlled method to inoculate, grow, stress, and sample *D. vulgaris* cultures.
- QA/QC verifications at every stage of biomass production insure maximum reproducibility between biomass production experiments.
- Centrifugation and the fast chilling system appropriately prepares replicate samples simultaneously for transcriptomics, proteomics, metabolomics, and lipidomics processing.
- The large scale biomass production of *Desulfovibrio vulgaris* for stress response studies can be used as a model for the large scale production of other obligate anaerobes in the future.
- The biomass production of batch cultures will provide valuable information and can be used for comparison when stressed *D. vulgaris* cells are produced in six replicate bioreactors in chemostat mode.

References

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